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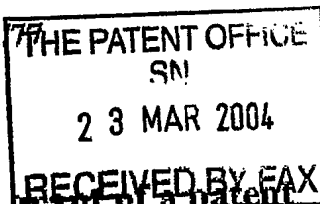
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Patents ADP number (if you know it)

7676620001

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4. Title of the invention **Methods**5. Name of your agent (if you have one)
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Continuation sheets of this form	0
Description	29
Claim(s)	9
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Priority documents

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Statement of inventorship and right to grant of a patent (Patents Form 7/77) 1

Request for preliminary examination and search (Patents Form 9/77)

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We request the grant of a patent on the basis of this application.

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DUPLICATE

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Methods5 **Technical Field**

The invention relates to live, whole cell assays for detecting Ca^{2+} influx using a detectable reporter, particularly a fluorescent reporter. The methods are useful for detection of compounds that modulate calcium influx and can be performed
10 in high throughput screening format.

Background to the Invention

Calcium influx into the cell from the extracellular medium is vital for processes
15 such as muscle contraction, secretion and gene activation. Calcium influx is mediated via calcium influx channels which can be divided into two groups on the basis of their activation mechanism: voltage-gated calcium channels and non-voltage gated calcium permeable calcium channels. Calcium influx can be stimulated or inhibited by factors that act on the calcium ion channel directly, or
20 indirectly where modulation of a receptor results in a signal that acts on a calcium ion channel.

Ion channels play an important role in numerous cell types and occur as large families of related genes. Ion channels, such as store-operated calcium
25 channels, receptor-operated and voltage-gated calcium channels are important selective tissue-specific targets for drug discovery. Currently more than a dozen ion channel drugs are marketed for the treatment of cardiovascular disease, diabetes, epilepsy and pain. Thus, there is interest in developing cell-based assays for screening compounds that modulate the activity of ion
30 channels and receptors associated with ion channels

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With the demand for screening large compound collections against an increasing number of therapeutic targets, there is a demand for improved assays, especially cell based assays and for such assays in automated, high throughput formats.

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The technology currently employed for ion channel screening includes, binding assays, fluorometric imaging and voltage sensing.

Whole cell functional assays are known for identification of compounds that act at plasma membrane ion channels or receptors (such as G-protein coupled receptors (GPCR)) and trigger calcium influx. The rapid increase in intracellular calcium concentration triggered by stimulation of these ion channels and receptors is detected using intracellular calcium probes; known probes include fluorescent dyes, or calcium-binding proteins. To detect the rapid, transient changes in intracellular calcium concentration on stimulation, a calcium influx stimulus is provided to cells and a change in fluorescence or luminescence from the calcium probe is detected. A problem with many of the methods for detecting the signal generated is of short duration and this restricts use of these assays in high throughput systems.

20

There have been attempts to delay or prolong the calcium signalling response to provide a greater temporal window for detection of calcium influx. One approach to prolong the calcium signalling response is described in US 6,514,709 in which an intracellular calcium chelating agent 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA-AM) was used to alter the kinetics of calcium signalling and delay or prolong the calcium signalling response.

25

A further problem is that assay methods using fluorescent Ca^{2+} dyes in cell lines expressing a Ca^{2+} channel of interest are not generally suitable for targeting to a specific sub-cellular compartment of interest, e.g. under the plasma membrane. The dyes are generally freely diffusible and give only a global indication of a Ca^{2+} change. Ca^{2+} dyes must be loaded into the cells, this can

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be done fairly consistently, but is inherently prone to differences between experiments. Generally, dyes do not report restricted spatial Ca^{2+} signals, e.g. extracellular Ca^{2+} influx induced by channel opening.

- 5 Another drawback of present assay methods is that intracellular agents used to detect calcium may adversely affect the behaviour of the cell, for example by buffering cytosolic calcium signals, and so the assay methods may not provide a true reflection of cellular events following calcium influx.
- 10 There is a desire to develop alternative whole cell assays using reporters sensitive to calcium influx.

The proteins RASAL and CAPRI (calcium promoted Ras inactivator) has been reported to be sensitive to increases in intracellular calcium due to ATP stimulation of calcium release from the endoplasmic reticulum. In resting cells RASAL and CAPRI is cytosolic and inactive. Following ATP stimulation, RASAL and CAPRI are translocated to the membrane. CAPRI is believed to

15 *Calcium Promoted Ras Inactivator* (CAPRI) (PCT/GB00/01499)

- 20 CAPRI is a member of the human GAP1 family of Ras GAPs (GAP1^{1013P}, GAP1^m, RASAL). Ras operates as a binary molecular switch, cycling between an inactive GDP-bound form and an active GTP-bound form at the plasma membrane or other intracellular membranes such as the cytosolic face of the Golgi. The intrinsic GDP/GTP exchange and GTPase activity of Ras is slow,
- 25 therefore control of GTPase cycling is modulated by guanine nucleotide exchange factors (GEFs) that promote the active GTP-bound state, and GTPase-activating proteins (GAPs) that promote the inactive GDP-bound state.

- Activated CAPRI inhibits the Ras/mitogen-activated protein kinase (MAPK) pathway by enhancing the intrinsic GTPase activity of Ras, resulting in
- 30 deactivation of Ras. Analysis of the spatio-temporal dynamics of CAPRI and RASAL indicates that calcium regulates Ras by a fast C2 domain-dependent translocation mechanism. Analysis was carried out in a whole cell assays in

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which RASAL, CAPRI and CAPRI mutants tagged with a green fluorescent protein (GFP) were expressed. ATP-induced release of calcium ions from the intracellular store induced a rapid translocation of RASAL and CAPRI to the plasma membrane and activation of CAPRI. This recruitment of RASAL and

5 CAPRI to the plasma membrane was detected because the GFP reporter resulted in acquisition of a fluorescent signal at the plasma membrane [1]

The human GAP1 Ras GAPs have a similar domain structure comprising of tandem C2 domains (C2A and C2B), a central GAP-related domain (GRD)
10 contiguous with a pleckstrin homology domain (PH domain) and Tec kinase homology domain (TH) near the C-terminus (Figure 1). Within the human GAP1 family, RASAL [3] is most closely related to CAPRI with 50% identity at the primary amino acid sequence level.

15 Statement of Invention

The present invention provides a method for detecting influx of calcium ions into a eukaryotic cell comprising providing a eukaryotic cell having a detectable
reporter capable of translocation from the cytosol to associate with the plasma
20 membrane in response to an influx of calcium ions, and monitoring association of the detectable reporter within the cell.

The present invention provides a method for detecting influx of calcium ions into a eukaryotic cell comprising providing a eukaryotic cell having a detectable
25 reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions, and, monitoring association of the detectable reporter with the plasma membrane and/or a change in amount of detectable reporter in the cytosol.

30 Both CAPRI and RASAL are Ca^{2+} -triggered Ras GAPs, they are located in the cytosol and are inactive in resting cells. An elevation in intracellular Ca^{2+} induces a rapid translocation of RASAL and CAPRI to the plasma membrane. This is mediated by tandem C2 domains (C2A C2B) that are known to be

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required for co-operative Ca^{2+} /phospholipid-binding in other C2 domain Ca^{2+} sensor proteins.

All known C2 domain containing Ca^{2+} sensors that do not constitutively
5 associate with cell membranes, e.g. through transmembrane domains,
contribute to the maintenance of repetitive Ca^{2+} signals such as
oscillations. Thus, RASAL and conventional protein kinase Cs (PKCs) oscillate
on and off the membrane in concert with repetitive Ca^{2+} (and diacylglycerol in
the case of PKC) signals. A major driver of complex oscillations in many cells is
10 so-called Ca^{2+} -induced Ca^{2+} release (CICR), a repetitive release of Ca^{2+} from
intracellular Ca^{2+} stores.

When intracellular stores are depleted as Ca^{2+} is released into the cytosol there
is a retrograde signal to the plasma membrane to open Ca^{2+} channels at the
15 cell surface, so-called capacitative or store-operated Ca^{2+} entry (SOCE) calcium
influx. This source of Ca^{2+} helps to replenish the intracellular Ca^{2+} stores and
maintain CICR. In addition, there are receptor-operated Ca^{2+} influx
channels that are activated by Ca^{2+} release from intracellular stores in
concert with, or in the absence of SOCE, in a stimulus- and cell type-specific
20 manner. Unlike Ca^{2+} release, Ca^{2+} influx is sustained in many cells, e.g.
lymphocytes, excitable cells and some secretory cells.

It has now been found that RASAL and CAPRI are differentially tuned to Ca^{2+}
signals. RASAL preferentially senses intracellular Ca^{2+} release, can oscillate
25 between the cytosol and membrane in concert with complex Ca^{2+} signals, and
therefore behaves as a frequency-modulated Ca^{2+} sensor. In contrast, CAPRI
preferentially senses Ca^{2+} influx, is refractory to cytosolic Ca^{2+} oscillations,
and therefore behaves like an analogue Ca^{2+} sensor of sustained Ca^{2+} influx.
Unlike all other known 'translocation sensors' of Ca^{2+} signals, CAPRI does not
30 sense oscillations of intracellular free Ca^{2+} . Instead, CAPRI senses SOCE and
is maintained at the plasma membrane for as long as SOCE occurs, even in the
presence of repetitive changes in cytosolic Ca^{2+} levels such as baseline spiking

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and sinusoidal oscillations. Translocation of a CAPRI reporter from the cytosol to the plasma membrane and maintenance at that location indicates that Ca^{2+} influx, specifically, is occurring.

- 5 The CAPRI and CAPRI derivatives have been modified so as to preferentially respond to Ca^{2+} influx into the cell.

Translocation of CAPRI to the plasma membrane is due to its' preferential
10 sensitivity to calcium influx rather than just release of calcium from the internal calcium store as initially thought. CAPRI and CAPRI derivatives can be used as calcium influx specific reporters useful in assay methods for detection of calcium influx into eukaryotic cells.

In methods of the invention, it is preferred that the detectable reporter is or
15 comprises CAPRI, or a derivative thereof which is capable of translocation to and association with the plasma membrane, labelled with a detectable substance.

20 particular to detect compounds that modulate calcium ion channels or that modulate receptors modulated by calcium ions. The present invention includes methods for identifying compounds that specifically interact with receptor polypeptides. Compounds that interact with a receptor can stimulate or inhibit the activity of a receptor. The term compounds as used herein includes chemically synthesised molecules and includes biological
25 molecules such as proteins and peptides.

The present invention provides method for identifying a compound capable of modulating influx of calcium ions into a eukaryotic cell comprising:

- 30 (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
(b) incubating the cell with a test compound, then
(c) providing a stimulus for calcium ion influx, and,

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- (d) monitoring association of the detectable reporter with the plasma membrane and/or a decrease in the detectable reporter in the cytosol.

In this aspect of the invention the cell is incubated with the test compound

and the association of the detectable reporter with the plasma membrane is monitored.

Also provided is a method for identifying a compound capable of modulating influx of calcium ions into a eukaryotic cell comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) providing a stimulus for calcium ion influx,
- (c) incubating the cell with a test compound,
- (d) monitoring dissociation of the detectable reporter with the plasma membrane and/or an increase in detectable reporter in the cytosol.

In this aspect of the invention, calcium influx into the cell is stimulated and then

the rate of association of the detectable reporter with the plasma membrane is monitored during calcium ion influx and the rate of dissociation of the reporter from the plasma membrane is monitored. The rate of association of the reporter with the plasma membrane is compared to the rate of dissociation of the reporter from the plasma membrane and the test compound is assessed.

A compound capable of modulating influx of calcium ions is a compound that alters the behaviour of an ion channel or receptor and is also termed a modulator, a modulator may be an antagonist, inhibitor or blocker that, directly or indirectly, decreases calcium influx through an ion channel. Alternatively the modulator can be an agonist or stimulator that, directly or indirectly, increases calcium influx through a calcium ion channel.

As used herein, an "agonist" is a molecule that stimulates an activity of an ion channel or receptor; an "antagonist" is a molecule that inhibits or interferes with the activation of an ion channel or receptor. An "inhibitor" or "blocker" is a

molecule prevents or reduces the opening of an ion channel or activation of a receptor.

The stimulus for calcium influx used in a method of the invention can be a stimulus or combination of stimuli for calcium influx, the stimulus can be one or more of the following:

- (a) agonist-evoked intracellular calcium release from intracellular stores, which indirectly stimulates calcium influx through SOCE,
- (b) agonist-evoked intracellular calcium release from intracellular stores, which stimulates calcium influx through NCCE,
- (c) agonist-evoked intracellular calcium release from intracellular stores which stimulates calcium influx through SOCE and NCCE,
- (d) chemical treatment leading to store release that activates SOCE, e.g. using the Ca^{2+} pump inhibitor thapsigargin,
- (e) an intracellular ligand that agonises a second messenger-operated plasma membrane Ca^{2+} channel,
- (f) an agent or conditions that cause depolarisation thereby opening a voltage-gated channel,
- (g) a stretch stimulus that opens a mechanosensitive channel,
- (h) a change in temperature sufficient to open a temperature-sensitive TRP family channel,
- (i) an agonist that opens a receptor-operated channel,
- (j) an extracellular ligand that opens a ligand-gated channel,
- (k) an intracellular or extracellular pH change that opens a redox-sensitive channel,
- (l) a change in osmolarity that opens an osmolarity-sensitive channel.

A G protein-coupled receptor such as a purinergic receptor can be stimulated using ATP, this causes Ca^{2+} release from internal stores which eventually triggers a signal for calcium influx (SOCE). Calcium ion channels can be opened by changes in lipid metabolism after phospholipase C activity induced by a GPCR or by the products of phospholipase C activity such as diacylglycerol (DAG). DAG has been shown to activate channels directly

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(TRPC3 and TRPC6) or through products of its breakdown, poly-unsaturated fatty acid metabolites such as arachidonic acid or linoleic acid (TRPL). TRPV channels can be activated using mechanical and physical stimuli such as low pH, heat and osmotic stress and ligands such as capsaicin and anandamide. (17) (18) (19) (20) Cells have been used to activate TRPV4 channels. In some cell lines such as HeLa and HEK293, TRPV4 can be activated directly by application of extracellular arachidonic acid through unknown channels (that may be TRP channels) termed arachidonate-regulated Ca^{2+} channels (ARC). Compounds other than thapsigargin that inhibit Ca^{2+} pumps leading to the depletion of intracellular store Ca^{2+} and reciprocal activation of GPCR (e.g. thapsigargin, 10 μM (GDA) and 0.5 μM (thapsigargin) (TRPC).

The invention includes a method for identifying a compound that modulates calcium ion influx comprising:

- 15 (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the membrane upstream in response to an influx of calcium ions,
- (b) monitoring association of the reporter with the membrane upstream of the reporter from cytosol while
- 20 (i) incubating the cell in conditions that stimulate calcium influx, and
- (ii) introducing a test compound to the incubation mixture, and,
- (c) comparing the association of the reporter with the membrane in the presence of the test compound with the association of the reporter with the membrane in the absence of the test compound,
- 25 wherein a change in the association of the reporter with the membrane in the presence of the test compound indicates that the test compound modulates calcium influx.

In this aspect of the invention, calcium influx into the cell is stimulated and then
30 the cell is exposed to the test compound. In a preferred embodiment, the cell is exposed to the test compound during calcium ion influx and the rate of dissociation of the reporter from the membrane verses the rate observed in a control cell(s) not exposed to the test compound is assessed.

The present invention provides methods useful as assays for identification of compounds that are receptor inhibitors (antagonists) or agonists.

5 In particular, the invention provides a method for identifying a compound that inhibits calcium ion influx comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- 10 (b) monitoring association of the reporter with the membrane, then
 - (i) incubating the cell in conditions that stimulate calcium influx, then
 - (ii) introducing a test compound to the incubation mixture, and,
- (c) comparing the association of the reporter with the membrane in the presence and absence of the test compound,
- 15 wherein a decrease in association of the reporter with the membrane following introduction of the test compound indicates that the test compound inhibits calcium ion influx.

In this aspect of the invention calcium influx into the cell is stimulated and then
20 the cell is exposed to the test compound during calcium ion influx. The rate of association of the reporter with the membrane is assessed while the cell is exposed to the test compound during calcium ion influx and the rate of dissociation of the reporter from the membrane verses the rate observed in a control cell(s) not exposed to the text compound is assessed.

25 Also provided is a method for identifying a compound that modulates calcium ion influx comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- 30 (b) providing a control by monitoring association of the reporter with the membrane while incubating the cell in conditions that stimulate calcium influx,
- (c) monitoring association of the reporter with the membrane while

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- (i) incubating the cell in the presence of a test compound, then
- (ii) incubating the cell in conditions that stimulate calcium influx, and
- (d) comparing the association of the reporter with the membrane in the absence and presence of the test compound,

wherein a change in association of the reporter with the membrane in the presence of the test compound compared to the association of the reporter with the membrane in the absence of the test compound indicates that the test compound modulates calcium influx.

In this aspect of the invention the cell is incubated with the test compound before calcium influx is stimulated.

Additionally the invention provides a method for identifying a compound that inhibits calcium ion influx comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) providing a control by monitoring association of the reporter with the membrane while incubating the cell in conditions that stimulate calcium influx,
- (c) monitoring association of the reporter with the membrane while
 - (i) introducing a test compound to the incubation mixture, then
 - (ii) incubating the cell in conditions that stimulate calcium influx, and
- (d) comparing the association of the reporter with the membrane in the absence and presence of the test compound

wherein a decrease in the association of the reporter with the membrane in the presence of the test compound compared to the association of the reporter with the membrane in the absence of the test compound indicates that the test compound inhibits calcium influx.

In this aspect of the invention the cell is incubated with the test compound in the presence of calcium ions before calcium influx is stimulated.

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In methods of the invention it is preferred that the cell (which may be a cell or cell line) is a mammalian cell. As used herein the term "cell" encompasses both cells (primary cells) and cell lines. Suitable cells include CHO, Cos, Jurkat-T, HeLa, PC12 or HEK293 cell.

5

In the methods of the invention, suitable conditions for incubation of the cells are those conditions generally used for whole cell assays. For example in specific embodiments, COS-7, HEK293 and HeLa cells can be transiently transfected with GFP-CAPRI plasmid DNA using Lipofectamine (GIBCO BRL)

10 a. Conduelise transfection reagent (Merieux) according to manufacturer's instructions. 40-50% confluent cells are transfected in EM buffer (10 mM NaCl, 5.4 mM KCl, 1.6 mM MgCl₂, 6 mM NaHCO₃, 9 mM glucose, 1.2 mM CaCl₂, 0.7

mM HEPES, pH 7.4) or KH buffer (10 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 10 mM glucose, 1.2 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.2 mM CaCl₂, pH 7.4).

15 For Ca²⁺-free conditions CaCl₂ can be replaced with 0.5 mM EGTA. To deplete intracellular Ca²⁺ stores prior to addback of Ca²⁺ containing media which leads to sustained SOCE across the plasma membrane and CAPRI translocation,

cells are incubated in Ca²⁺-free media for 15-30 minutes (volumes relevant to 22 mm coverslips and holder). Addition of 5 ml of

20 Ca²⁺-containing media causes sustained Ca²⁺ entry across the plasma membrane.

To stimulate calcium influx, an agonist such as histamine (HeLa; 1-100 μ M) or ATP (HeLa, HEK293 and COS; 50 μ M) can be applied by bulk addition (rapid mixing of 5 ml of agonist in appropriate imaging buffer e.g. EM or KH buffer as above) added to 2 ml of buffer containing the coverslip with vacuum line attachment to maintain total volume of 2 ml). These agonists activate SOCE and/or NSOCE. Histamine stimulation of HeLa cells can lead to sustained (>15 minutes) translocation of GFP-CAPRI to the plasma membrane at

30 supramaximal agonist doses.

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Live imaging in low throughput format is suitably performed at 25°C or 37°C on an inverted microscope (Nikon) attached to a PerkinElmer LCI or RS confocal imaging system.

- 5 The present invention can be applied generally to whole cell functional assays for any calcium channel, or any receptor or ion channel that is coupled to a calcium channel. Suitably, a cell used in a method of the invention is capable of expressing, endogenously and/or ectopically, one or more of the calcium channels selected from the group comprising: TRP family channels, voltage-gated channels, ligand-gated channels, receptor-operated channels, Ca^{2+} release channels, Ca^{2+} store-operated channels, or any combination of the foregoing channels. By "endogenously" it is meant that the channel(s) is/are normally encoded by the cell, i.e. that the channels are present in the native cell. By "ectopically" it is meant that the channel or
- 10 channels is/are expressed from nucleic acid that has been introduced into the cell and is either stably integrated into the genome or is present extrachromosomally, e.g. following transient transfection with a vector(s) encoding the nucleic acid encoding the channel(s).
- 20 Cells used in the methods of the invention have a calcium influx sensitive detectable reporter. In certain embodiments of the invention, the detectable reporter is a protein expressed within the cell. The cell can be engineered to express the detectable reporter protein from stably integrated nucleic acid or for ectopic expression, the cell can be stably or transiently transfected with nucleic acid encoding the detectable reporter protein, suitably the nucleic acid encoding the detectable reporter protein is comprised within an expression vector.
- 25

- In alternative embodiments of the methods, the cells do not express the calcium influx sensitive detectable reporter and instead, the reporter is introduced into
- 30 the cell for purposes of conducting the assay, e.g. by permeabilisation, by using lipid reagents, or microinjection. This embodiment can be useful with cells that endogenously or ectopically express an ion channel(s) or a receptor(s) that one

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desires to use in the assay. Thus as an alternative to expressing the detectable reporter within the cell, the detectable reporter can be introduced into the cell.

5 In preferred methods, the detectable reporter is labelled with a fluorescent marker.

A suitable detectable reporter for use in methods where the reporter is expressed within the cell, or in methods where the reporter is introduced into the cell is a protein chimera having a calcium influx sensitive reporter moiety
10 and a fluorescent protein moiety.

The fluorescent protein moiety may be, for example, "enhanced yellow", yellow, green or blue (cyan) fluorescent protein. The fluorescent protein may be a wild type, enhanced, destabilised enhanced, or red shift fluorescent protein.

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As an alternative to using a single fluorescent protein, a pair of fluorescent proteins may be used to detect the location of the reporter. For example, FRET between a plasma membrane localised fluorophore and a calcium influx sensitive fluorescent reporter may be used to detect the location of the reporter.

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FRET between a plasma membrane localised fluorophore and a calcium influx sensitive fluorescent reporter may be used to detect the location of the reporter. For example, a YFP-CAPRI or CAPRI derivative reporter, the YFP-CAPRI can be endogenously or ectopically expressed within the cell or introduced into the cell.

25

Other suitable detectable reporters are those in which a calcium influx sensitive reporter protein moiety is either expressed within the cell, or introduced into the cell and the reporter is labelled *in vivo*, i.e. within the cell, with a fluorescent moiety which is introduced into the cell. Such detectable reporters include: a detectable reporter in which the C-terminus of the reporter is fused to ^{W180}hAGT (O⁶-alkylguanine-DNA alkyltransferase) which is fluorescently labelled following
30 a reaction with O⁶-benzylguanine fluorescein (BGFL); and a detectable reporter in which the reporter a tetracysteine motif is added to the N- or C-terminus of the reporter and to which a bi-arsenic fluorophore is covalently linked ('FIAsh labelling').

Other fluorescent detectable reporters cannot be expressed within the cell, but are suitable for use in methods of the invention where the calcium influx sensitive detectable reporter is introduced into the cell for example a detectable reporter in which the reporter protein is labelled with a fluorophore, for example a small organic fluorophore, e.g. fluorescein or rhodamine; or a quantum dot (Q dot).

In a preferred embodiment of the invention, the reporter is labelled with a fluorescent marker which is a quantum dot (Quantum dot comprising). Quantum dots are tiny particles of semiconductor material, typically only a few nanometres in diameter, which have the property of absorbing light at one wavelength but then re-emitting light at a different wavelength, usually at a higher frequency. The size of the dot determines the colour of light that it emits: a 2 nanometre dot emits green light, while a 5 nanometre dot emits red light. The reporter can be directly labelled with the quantum dot and introduced into the cell. Alternatively, the reporter can be labelled with the quantum dot by using a "kitchen-sink" method, in which the quantum dot is first attached to a biotin molecule, and then the biotin-labelled quantum dot can be introduced into the cell. As a further alternative using a quantum dot as the detectable marker, the quantum dot can be attached to a biotin molecule, and then the biotin-labelled quantum dot can be introduced into the cell.

In preferred methods the detectable reporter is or comprises CAPRI, or a derivative thereof which is capable of association with the plasma membrane, labelled with a detectable marker.

A CAPRI reporter according to present invention is generally applicable for detecting and measuring calcium influx of calcium whether by fluorescence, luminescent or other detection techniques, depending on the detectable marker employed.

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In preferred methods of the invention, the detectable reporter is or comprises CAPRI, or a derivative thereof which is capable of association with the plasma membrane, labelled with a fluorescent protein, e.g. GFP-CAPRI.

5 A CAPRI derivative suitable for use in a method of the invention may be a mutated and/or truncated CAPRI that is capable of translocation to the plasma membrane. A CAPRI derivative is suitably a mutated CAPRI, having a mutation such that it no longer acts as a Ras GAP. A suitable mutant derivative of CAPRI is R473S [2], this derivative is advantageous because it translocates to the plasma membrane in response to Ca^{2+} influx but does not affect Ras, and
10 thus its primary function is calcium influx sensing. In the R473S CAPRI mutant, the primary function of CAPRI is calcium influx sensing, and the CAPRI derivative is a CAPRI derivative.

15 Other CAPRI derivatives useful as reporters in methods of the invention include mutated and/or truncated derivatives of CAPRI, e.g. those that increase or decrease the sensitivity to Ca^{2+} entry by altering the kinetics of translocation/association of CAPRI with the plasma membrane. CAPRI derivatives for use in methods of the invention include C2A, C2B domains (tandem C2 domains) that primarily sense Ca^{2+} signals translocating with the
20 plasma membrane. Thus a further useful CAPRI derivative is a truncated derivative of CAPRI that is or comprises the tandem C2 domain C2A, C2B, (Met 1 to Leu 275 or CAPRI), and in which the domain(s) may be in wild type or mutated form. Individually, the single domains of CAPRI have not been found
25 to be suitable for use as reporters in methods of the invention, as neither the C2A (Met 1 – Glu 141), nor the C2B domain (Glu 119 – Leu 275) will translocate to the plasma membrane in response to an influx of calcium ions.

30 Fluorescent protein chimeras comprising CAPRI, or a derivative thereof which is capable of association with the plasma membrane and a fluorescent protein, e.g. green fluorescent protein, can be used as genetically-encodable reporters of Ca^{2+} influx, suitable for use in methods of the invention.

Constructs encoding the CAPRI reporter can be transfected into cell lines by standard techniques e.g. electroporation, Ca^{2+} phosphate, lipofection, gene gun. Recombinant retroviruses, adenoviruses or lentiviruses can also be used to introduce genetic material encoding the reporter CAPRI into cells by infection. Selection of cells expressing a CAPRI fluorescent protein (FP) or FP-CAPRI reporter can be made by FACS. Alternatively, selection of cells expressing an antibiotic resistance gene carried by the vector can provide a means for selection of transformed cells.

Alternatively a FRET method can be employed, for example by the use of a YFP-CAPRI construct. YFP-CAPRI can be used to monitor the association of CAPRI with a fluorescent protein for targeting to the plasma membrane. YFP-CAPRI or YFP-CAPRI derivative encoding the YFP-CAPRI can be expressed within the cell or introduced into the cell.

The invention thus provides a method for identifying a compound that modulates calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing a CAPRI or a derivative thereof which is capable of translocation to and association with the plasma membrane,
 - (b) incubating the eukaryotic cell in the presence of a test compound,
 - (c) monitoring fluorescence of the cell cytosol and/or plasma membrane,
- wherein a change in fluorescence in the cytosol and/or at the plasma membrane following addition of the test compound is indicative that the test compound modulates calcium ion influx.

The invention further provides a method for identifying a compound that stimulates (agonises) calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing FP-CAPRI or a derivative thereof, which is capable of translocation to and association with the plasma membrane,
- (b) incubating the eukaryotic cell in the presence of a test compound,
- (c) monitoring fluorescence of the cell cytosol, and/or plasma membrane

wherein a decrease in cytosolic fluorescence, and/or increase in plasma membrane fluorescence following addition of the test compound is indicative that the test compound stimulates (agonises) calcium ion influx.

5 Also provided is a method for identifying a compound that inhibits (antagonises) calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing EP-CARD1 or a derivative thereof which is capable of translocation to and association with the plasma membrane,
- 10 (b) providing a stimulus for calcium ion influx,
- (c) monitoring fluorescence of the cell cytosol and/or plasma membrane
- (d) incubating a test compound to the test buffer solution,
- (e) monitoring fluorescence of the cell cytosol and/or plasma membrane

15 wherein an increase in cytosolic fluorescence and/or a decrease in plasma membrane fluorescence following addition of the test compound is indicative that the test compound inhibits (antagonises) calcium ion influx.

Further provided is a method for identifying a compound that agonises (stimulates) calcium ion influx comprising:

- 20 (a) providing a eukaryotic cell expressing EP-CARD1 or a derivative thereof which is capable of translocation to and association with the plasma membrane,
- (b) incubating the eukaryotic cell in the presence of a test compound,
- (c) monitoring fluorescence of the cell cytosol and/or plasma membrane,
- 25 wherein a decrease in cytosolic fluorescence and/or an increase in plasma membrane fluorescence following addition of the test compound is indicative that the test compound agonises (stimulates) calcium ion influx.

30 An advantage of the methods of the invention is that the signal following calcium influx is prolonged, so that the signal can be readily and reliably detected by automated instrumentation.

The present invention advantageously provides for the use of instrumentation to

detect fluorescent or luminescent signals from cells. In preferred methods the detectable marker is fluorescent and monitoring is performed by fluorescence microscopy. Fluorescence microscopy can be performed using wide-field or total internal reflection fluorescence microscopy (TIRF) or fluorescence lifetime
5 imaging or confocal imaging.

In methods of the invention, cells having the fluorescent reporter can be imaged live, or fixed at a given time point, but preferably cells are imaged live, cells are preferably monitored using a HT imaging device, e.g. Amersham INCell
10 imager.

In methods of the invention, monitoring can be performed by measuring fluorescence at the region(s) of interest within the cell over time. Association of the reporter with the plasma membrane and/or decrease of reporter in the
15 cytosol can be assessed using any suitable algorithm or equation, an example is by calculating the relative translocation parameter (TRTP) at two or more time points, wherein Fo is the fluorescence in a region(s) of interest (e.g. cytosol or plasma membrane) at the start of monitoring and Ft is the fluorescence in the region(s) of interest at a later time point or points.

20 During monitoring, readings should be made as often as possible, ideally at intervals of less than 10 seconds; the length of time over which monitoring is performed will vary with the nature of the channel being analysed. Monitoring may be performed for time periods of from several seconds, to up to an hour.
25 Suitably, readings may be taken every 5, 10, 15, 20 or 30 seconds over time periods of 5, 10, 20, 30 or 60 minutes. The frequency of readings and time period for monitoring can be experimentally determined and readily optimised for a particular assay, i.e. for particular cells/ion channels. For example, using HeLa cells stimulated with histamine, readings are taken every 4 or 5 seconds
30 for up to 15 minutes.

Monitoring can be performed by measuring cytosolic fluorescence over time as assessed by calculating the relative translocation parameter at one or more

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time points, $1 - F_{t_{\text{cyt}}} / F_{o_{\text{cyt}}}$, wherein $F_{o_{\text{cyt}}}$ is the cytosolic fluorescence in the region of interest at the start of monitoring and $F_{t_{\text{cyt}}}$ is the cytosolic fluorescence in the region of interest at a particular time point. A decrease in cytosolic fluorescence results in an increase in relative translocation parameter).

5

Methods of the invention can be performed in a high throughput format.

Many functional assays for calcium signalling are termed "flash" assays because they are instantaneous and transient as they are complete within a few seconds of the calcium ion influx stimulus, however in methods of the invention calcium ion influx can be detected for prolonged periods of tens of minutes up to several hours.

10

The invention is advantageously employed in whole cell functional assays and high throughput screening assays for calcium signalling such as calcium channels and receptors such as GPCRs to extend the signal so that it could be read by conventional plate readers.

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Methods of the invention are also performed in a high throughput format, for example in 96, 384, or 1536 multiwell plates or other plate formats. In a preferred embodiment, the assay is conducted in a multi well plate format and an instrument is used for monitoring in each well. The multiwell plates can be handled over an extended time period because of the extended time period over which calcium influx can be detected.

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The invention provides methods for identification of calcium channel modulating compounds, preferably in high throughput screening (HTS) format, using cells having a reporter derived from the protein CAPRI (e.g. fluorescent protein tagged CAPRI or CAPRI derivative) which is expressed by the cell or introduced into the cell.

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In a method of the invention, cells expressing a fluorescent protein-CAPRI reporter (FP-CAPRI), or a derivative thereof are incubated in the presence of a

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test compound, the effect of the test compound on calcium channels (block/activation) can be detected using FP-CAPRI as a reporter for calcium influx; with translocation of the CAPRI reporter to the membrane and maintenance at that locus being indicative of influx. To detect compounds that
5 block influx, the cells are subjected to a calcium influx stimulus.

In preferred method of the invention CAPRI is genetically encoded, and lines of interest can be cloned to stably express this reporter, allowing consistency between experiments. Translocation is specifically sensitive to
10 Ca^{2+} influx across the plasma membrane and CAPRI is a sensitive and specific marker is sensitive and specific indicator of Ca^{2+} channel activation/block.

Cells having the fluorescent reporter are imaged live, or fixed at a given time point (but preferably live) preferably using a HT imaging device, e.g. Amersham
15 InCell analyzer.

The methods of the invention are ideal for use in HTS to identify new calcium ion channel blockers. The method can be performed using currently available multi-well imaging platforms.
20

Methods of the invention are usually performed such that the cells are incubated throughout the method in media containing calcium ions. However, in some embodiments of methods of the invention cells are initially incubated in calcium-free media and in the presence of a compound that enhances the
25 release and/or leak of calcium from the intracellular store prior to exposure of the cells to the test compound and/or calcium influx stimulus; in methods that employ this treatment, calcium is then added back to the incubation so that influx can be detected.

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List of Figures

Figure 1, Molecular architecture of CAPRI and RASAL, with percentage identity between RASAL and CAPRI indicated. Each protein consists of C2A and C2B domains, a central GAP-related domain (GRD), and a pleckstrin homology (PH), RasGEF domain (Fig.).

Figure 2A, Relative translocation parameter. Each image was background corrected prior to measuring the average pixel intensity of a given region of interest (ROI) corresponding to 1/4 of the cell area. The parameter $T = (I_{ROI} - I_{cyt}) / (I_{PM} - I_{cyt})$ was calculated as the ratio of the intensity of the translocation of a given GFP chimera to the plasma membrane.

Figure 2B, Translocation of CAPRI and RASAL in response to 100 μ M histamine stimulation of HeLa cells. Time (t) = seconds after histamine stimulation. Translocation of GFP-CAPRI is sustained, while GFP-RASAL is transient and oscillatory in parallel experiments. GFP CAPRI is detectable at the plasma membrane for up to 15 minutes after stimulation. Time course of experimental monitoring performed, taking into account photobleaching of GFP, with an acquisition rate of 15 frames/second, 2.0 μ m²/frame, and a dwell time of 1 second per image on the PerkinElmer RS confocal microscope.

Figure 2C Kinetics of CAPRI and RASAL translocations. RASAL (bottom) oscillates between the plasma membrane and cytosol in phase with cytosolic Ca^{2+} oscillations [4]. Average n=3 cells. In contrast, CAPRI (top) never oscillates in phase with repetitive Ca^{2+} signals in the cytosol. Instead, translocation to the plasma membrane is sustained. Average n=7 cells from n=6 experiments for 100 μ M histamine, n=5 cells from n=3 experiments for 10 μ M histamine.

Figure 2D CAPRI and RASAL translocations in COS-7 cells stimulated with 50 μ M ATP. The rate of RASAL translocation matches the release of Ca^{2+} from intracellular stores. In contrast the rate of CAPRI translocation (grey line) is slow

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and more sustained. This is likely to reflect the fact that CAPRI, operating as a Ca^{2+} influx sensor, is sensitive to the latency of SOCE/NSOCE induced by purinergic stimulation in this cell type. Experiment is an average of $n=6$ cells for GFP-CAPRI and $n=9$ cells from 2 experiments for GFP-RASAL. Methods as for Figure 2B.

Figure 3 Representative traces of cytoplasmic Fura-2 and GFP imaging of Ca^{2+} in cells expressing GFP-CAPRI. Fura-2 emission was monitored at 380 nm excitation only (non-ratiometric) to track changes in cytosolic Ca^{2+} concentration. Ca^{2+} entry was monitored by Ca^{2+} imaging of GFP-CAPRI translocation (black line). GFP-CAPRI translocation (grey line) is measured as the Ratio of Translocation according to Figure 2A. $10 \mu\text{M}$ thapsigargin (100 ng/ml) generates sinusoidal Ca^{2+} oscillations or base line Ca^{2+} spikes. GFP-CAPRI is refractory to repetitive Ca^{2+} signals unlike RASAL [4] which is entirely consistent with the preferential sensing of Ca^{2+} entry by CAPRI.

Figure 4 CAPRI sensitivity to SOCE. GFP-CAPRI (black trace) and GFP-RASAL (grey trace) expressing HeLa cells were treated with $1 \mu\text{M}$ thapsigargin in a buffer media containing $10 \mu\text{M}$ CaCl_2 and $10 \mu\text{M}$ EGTA. Ca^{2+} entry (grey trace) was monitored by Ca^{2+} imaging of GFP-CAPRI (black trace). $10 \mu\text{M}$ thapsigargin (100 ng/ml) was added by bulk addition. In the period that RASAL takes to fully dissociate from the membrane CAPRI association is maintained by store-operated Ca^{2+} entry through SOCE channels. Methods as Figure 2B.

References

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Examples

Example 1: Ca^{2+} influx assay using a genetically-encoded fluorescent reporter - translocation of CAPRI and RASAL in response to agonist (100 μM histamine) stimulation of HeLa cells.

The day before transfection HeLa cells (1×10^6) were seeded on glass coverslips in 6-well tissue culture flasks at a density to reach 80-90% confluence within 24 hours. The following day (approximately 24 hours later) 10 1×10^6 cells were transfected with the construct encoding GFP-CAPRI or GFP-RASAL using GeneJuice Transfection Reagent (Mirus) according to the manufacturer's instructions.

After 24 hrs incubation in transfection mix (complete media plus transfection 15 reagent) at 37°C, 5% CO_2 coverslips were transferred to holders containing 2 ml of KH buffer (10 mM HEPES, 119 mM NaCl, 1.7 mM KCl, 12 mM glucose, 1.2 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 1.2 mM CaCl_2 , pH 7.4) and monitored as described by live imaging in a 37°C heated chamber on an inverted Nikon TE 2000 microscope using a 40x oil objective lens. Individual images were acquired every 10 seconds for 15 minutes. The images were then processed using a 2x2 binning, using a PerkinElmer RS confocal system with a 403 nm laser line.

Rapid mixing of the agonist histamine was achieved by bulk addition of 5 ml of 25 37°C KH media plus histamine, with a vacuum line maintaining a maximum volume of 2ml within the coverslip holder.

For calculation of the relative translocation parameter, each image was background corrected prior to measuring the average pixel intensity of a given region of interest (ROI) corresponding to >10% of the cytosolic area (see Figure 30 2A). The Relative Translocation (RT) parameter was calculated, $\text{RT} = 1 - F/F_0$, to provide an indication of the translocation of the GFP-CAPRI or GFP-RASAL chimera to the plasma membrane.

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Translocation of CAPRI and RASAL in response to agonist stimulation of calcium influx into HeLa cells using 100 μ M histamine is shown in Figure 2B. Time (t) = seconds after histamine stimulation. Translocation of GFP-CAPRI and maintenance at the plasma membrane is sustained, whereas GFP-RASAL is transient and oscillatory in parallel experiments. GFP-CAPRI is detectable at the plasma membrane for up to 10 minutes after stimulation. The maximum value was 5, a maximum length of the cytoplasmic tail of 500 nm, the translocation of GFP with an acquisition rate of 45 frames/second, 200 binning, and an exposure time of 1 second per image on the PerkinElmer RS confocal microscope.

The kinetics of CAPRI and RASAL translocation in response to histamine is shown in Figure 2C, which the relative translocation parameter is plotted against time. RASAL (bottom) oscillates between the plasma membrane and cytosol in phase with cytosolic Ca^{2+} oscillations [4]. Average n=3 cells. In contrast, CAPRI (top) never oscillates in phase with repetitive Ca^{2+} signals in the cytosol. Instead, translocation to the plasma membrane is sustained. Average n=7 cells from n=6 experiments for 100 μ M histamine, n=5 cells from n=3 experiments for 10 μ M histamine.

CAPRI and RASAL translocations in COS-7 cells stimulated with 50 μ M ATP (to release calcium ions from the intracellular store) are shown in figure 2D. The rate of RASAL translocation matches the release of Ca^{2+} from intracellular stores. In contrast the rate of CAPRI translocation (grey line) is slow and more sustained. This indicates that CAPRI, operating as a Ca^{2+} influx sensor, is sensitive to the latency of SOCE/NSOCE induced by purinergic stimulation in this cell type. The experiment is an average of n=6 cells for GFP-CAPRI and n=9 cells from 2 experiments for GFP-RASAL. The methods used were as described above.

Representative traces of sequential Fura-2 and GFP imaging of HeLa cells expressing GFP-CAPRI over 600 seconds (top) and 330 seconds (bottom) are shown in Figure 3. Fura-2 emission was monitored at 380 nm excitation only

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(non-ratiometric) to track changes in cytosolic Ca^{2+} concentration, expressed as the inverse change in fluorescence intensity at 380 nm excitation (black line). GFP-CAPRI translocation (grey line) is expressed as the Relative Translocation calculated as above. Application of 10 μM histamine generates sinusoidal Ca^{2+} oscillations or baseline Ca^{2+} spikes. GFP-CAPRI is refractory to small Ca^{2+} oscillations or spikes, but is sensitive to the sustained rise in Ca^{2+} concentration following sensing of Ca^{2+} influx by CAPRI.

CAPRI sensitivity to SOCE is shown in Figure 4. In this endback protocol to demonstrate SOCE, the method is used to monitor the translocation of GFP-CAPRI (black line) as GFP-CAPRI translocation is a sensitive indicator of Ca^{2+} concentration. Cells are grown in DMEM containing 10% FBS and 0.5 mM ECTA (nominally zero Ca^{2+} containing media); then, after 10 seconds, 5 ml of KH media/1 μM thapsigargin (1.3 mM CaCl_2 KH buffer) was added by bulk addition, providing Ca^{2+} ions. Cells are monitored at 380 nm and readings taken every 5 seconds for up to 15 minutes. In this protocol, CAPRI takes to fully dissociate from the membrane. CAPRI translocation is inhibited by store operated Ca^{2+} entry through SOCE after cells are exposed to thapsigargin, which blocks release of calcium ions from the intracellular store (ER).

Example 2: Screening to detect a receptor antagonist compound.

The day before transfection COS-7, HEK293 and HeLa cells are seeded onto 22 mm glass coverslips in 6-well tissue culture dishes at such a density to reach 60-80% confluence within 24 hours. After approximately 24 hours, COS-7, HEK293 and HeLa cells are transiently transfected with GFP-CAPRI plasmid DNA using Lipofectamine (GIBCO BRL) or GeneJuice transfection reagent (Merck) according to manufacturers instructions and incubated for 24 hours as above. The cells are incubated with test compound in EM buffer (121 mM NaCl, 5.4 mM KCl, 1.6 mM MgCl_2 , 6 mM NaHCO_3 , 9 mM glucose, 1.3 mM CaCl_2 , 25 mM HEPES, pH 7.4) or KH buffer (10 mM HEPES, 118 mM NaCl,

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Prior to introduction of the test compound, cells are stimulated with 1-5 μM thapsigargin in 2 ml Ca^{2+} -free media for 5 minutes. The cells are then incubated in the presence of the test compound for up to one hour.

- 5 The cells are imaged in Ca^{2+} -free EM buffer (101 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl_2 , 1.0 mM CaCl_2 , 10 mM HEPES, 110 mM NaCl, 1.7 mM MgCl_2 , 1.0 mM glucose, 1.2 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 0.5 mM EGTA, pH 7.2) or KH buffer (10 mM HEPES, 110 mM NaCl, 1.7 mM MgCl_2 , 1.0 mM glucose, 1.2 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 0.5 mM EGTA, pH 7.2) during and after Ca^{2+} addback, by bulk addition of 5 ml of EM or KH buffer containing Ca^{2+} to the 2 ml of Ca^{2+} -free buffer containing the coverslip. A vacuum line attachment was used to maintain total volume of 2 ml.

- 10 Live imaging was performed at 25°C or 37°C, before, during and after Ca^{2+} addback on an inverted microscope (Nikon) attached to a PerkinElmer LCI or RS confocal imaging system. Readings are taken every 5 seconds for 15 minutes.

- 15 Failure to observe translocation of the CAPRI reporter was observed for cells transfected with CAPRI plasmid lacking C-terminal domain (CAPRI- ΔC), and cells transfected with CAPRI plasmid lacking N-terminal domain (CAPRI- ΔN).

20 Example 4: Screening for SOCE channel inhibitors.

- The day before transfection COS-7, HEK293 and HeLa cells are seeded onto 25 22 mm glass coverslips in 6-well tissue culture dishes at such a density to reach 60-80% confluence within 24 hours. After approximately 24 hours, COS-7, HEK293 and HeLa cells are transiently transfected with GFP-CAPRI plasmid DNA using Lipofectamine (GIBCO BRL) or GeneJuice transfection reagent (Merck) according to manufacturers instructions and incubated for 24 hours as 30 above.

The cells are incubated with 1-5 μM thapsigargin in 2 ml Ca^{2+} -free EM or KH media for 5 minutes. Ca^{2+} addback is then carried out by bulk addition of 5 ml

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of EM or KH buffer (as appropriate) containing Ca^{2+} to the 2 ml of corresponding Ca^{2+} -free buffer containing the coverslip. A vacuum line attachment was used to maintain total volume of 2 ml.

- 5 The test compound is added to cells 30 seconds after Ca^{2+} attachment by buffer compound.

- Live imaging is performed at 25°C or 37°C before, during and after addition of the test compound using a hybrid video scope (MRC) attached to a PerkinElmer LCI or RS confocal imaging system. Readings are taken every 7 seconds for 15 minutes.

- An increase in the rate of GFP-CAPRI dissociation from the membrane compared to control cells indicates the effect of Ca^{2+} entry blockade by the test compound.

Experimental Results

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Genebank accession numbers for CAPRI (RASA4) are NM000000 and AY029206. Genebank accession number for ELASAL is NM004652.

25

CLAIMS:

1. A method for detecting influx of calcium ions into a eukaryotic cell comprising providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions, and monitoring association of the detectable reporter with the plasma membrane and/or a decrease in the detectable reporter in the cytosol.
2. A method for identifying a compound capable of modulating influx of calcium ions into a eukaryotic cell comprising:
 - (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
 - (b) incubating the cell with a test compound,
 - (c) providing a stimulus for calcium influx, and,
 - (d) monitoring association of the detectable reporter with the plasma membrane and/or a decrease in the detectable reporter in the cytosol.
3. A method for identifying a compound capable of modulating influx of calcium ions into a eukaryotic cell comprising:
 - (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
 - (b) providing a stimulus for calcium influx,
 - (c) incubating the cell with a test compound,
 - (d) monitoring association of the detectable reporter with the plasma membrane and/or a decrease in detectable reporter in the cytosol.
4. A method for identifying a compound that modulates calcium ion influx comprising:

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- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
 - (b) as a control, monitoring association of the reporter with the membrane, and/or loss of reporter from cytosol while incubating the cell in conditions that stimulate calcium influx, then
 - (c) monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while
 - (i) incubating the cell in conditions that stimulate calcium influx, then
 - (ii) introducing a test compound to the incubation mixture, and,
 - (d) comparing the association of the reporter with the membrane and/or loss of reporter from cytosol in the presence of the test compound with the association of the reporter with the membrane and/or loss of reporter from cytosol in the absence of the test compound,
- wherein a difference in the association of the reporter with the membrane and/or loss of reporter from cytosol in the presence of the test compound indicates that the test compound modulates calcium influx

5. A method for identifying a compound that acting as a calcium influx modulator comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) as a control, monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while
 - (i) incubating the cell in conditions that stimulate calcium influx,
- (b) monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while
 - (i) incubating the cell in conditions that stimulate calcium influx, then,
 - (ii) introducing a test compound to the incubation mixture, and,
- (c) comparing the association of the reporter with the membrane and/or loss of reporter from cytosol in the presence and absence of the test compound,

wherein a decrease in association of the reporter with the membrane and/or an increase in reporter in the cytosol following introduction of the test compound indicates that the test compound antagonises calcium ion influx.

6. A method for identifying a compound that agonises calcium ion influx

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) monitoring association of the reporter with the membrane and/or loss of reporter from the cytosol while incubating the cell in conditions that stimulate calcium influx, and
- (c) comparing the association of the reporter with the membrane and/or loss of reporter from cytosol in the absence and presence of the test compound,

wherein an increase in association of the reporter with the membrane and/or a decrease in reporter in the cytosol following introduction of the test compound indicates that the test compound agonises calcium ion influx.

7. A method for identifying a compound that antagonises calcium ion influx comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) providing a control by monitoring association of the reporter with the membrane while incubating the cell in conditions that stimulate calcium influx,
- (c) monitoring association of the reporter with the membrane while
 - (i) incubating the cell in the presence of a test compound, then
 - (ii) incubating the cell in conditions that stimulate calcium influx, and
- (d) comparing the association of the reporter with the membrane in the absence and presence of the test compound,

wherein a difference in association of the reporter with the membrane and/or a difference in loss of reporter from cytosol in the presence of the test compound compared to that in the absence of the test compound indicates that the test compound modulates calcium influx.

comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) providing a control by monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while incubating the cell in conditions that stimulate calcium influx,
- (c) monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while
 - (i) introducing a test compound to the incubation mixture, then
 - (ii) incubating the cell in conditions that stimulate calcium influx, and
- (d) comparing the association of the reporter with the membrane and/or loss of reporter from cytosol in the absence or presence of the test compound

wherein a decrease in the association of the reporter with the membrane and/or a decreased in loss of reporter from cytosol in the presence of the test compound compared to that in the absence of the test compound indicates that the test compound antagonises calcium influx.

9. A method for identifying a compound that agonises calcium ion influx comprising:

- (a) providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an influx of calcium ions,
- (b) providing a control by monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while incubating the cell in conditions that stimulate calcium influx,

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- (c) monitoring association of the reporter with the membrane and/or loss of reporter from cytosol while
 - (i) introducing a test compound to the incubation mixture, then
 - (ii) incubating the cell in conditions that stimulate calcium influx, and
- (d) comparing the association of the reporter with the membrane and/or loss of reporter from cytosol with and without the test compound

wherein a increase in the association of the reporter with the membrane and/or a increase in loss of reporter from cytosol in the presence of the test compound compared to that in the absence of the test compound indicates that the test compound agonizes calcium influx.

10. A method according to any preceding claim wherein the cell is a mammalian cell.

11. A method according to any preceding claim wherein the cell is a CHO Cos, Jurkat-T, HeLa, PC12 or HEK293 cell.

12. A method according to any preceding claim wherein the detectable reporter is expressing, endogenously or ectopically, one or more of the following channels selected from the group comprising: TRP family channels, voltage-gated channels, ligand-gated channels, receptor-operated channels.

13. A method according to any preceding claim wherein the detectable reporter is expressed within the eukaryotic cell.

14. A method according claim 13 wherein a nucleic acid encoding the detectable reporter is stably integrated within the eukaryotic cell.

15. A method according to any one of claims 1 to 13 wherein a nucleic acid encoding the detectable reporter is transiently transfected into the eukaryotic cell.

16. A method according to any one of claims 1 to 12 wherein the detectable reporter is introduced into the cell.
17. A method according to any preceding claim wherein the detectable reporter is or comprises CAPRI, or a CAPRI derivative, or a CAPRI derivative comprising a detectable marker.
18. A method according to any preceding claim wherein the detectable reporter is or comprises the CAPRI domain of CAPRI, or a CAPRI derivative comprising a detectable marker.
19. A method according to any preceding claim wherein the detectable reporter is a CAPRI derivative comprising or consisting of the CAPRI C2A C2D domains, with wild type or mutated sequence, labelled with a detectable marker.
20. A method according to any preceding claim wherein the detectable reporter is a reporter labelled with a fluorescent marker.
21. A method for identifying a compound which is capable of inhibiting translocation comprising:
- (a) providing a eukaryotic cell expressing FF CAPRI or a FF CAPRI derivative which is capable of translocation to and association with the plasma membrane;
- (b) (i) as a control, incubating the eukaryotic cell in the absence of test compound then providing a stimulus for calcium influx, and,
- (ii) incubating the eukaryotic cell in the presence of a test compound, then providing a stimulus for calcium influx; or;
- (c) (i) as a control, incubating the eukaryotic cell and providing a stimulus for calcium influx in the absence of test compound, and,
- (ii) incubating the eukaryotic cell and providing a stimulus for calcium influx, then providing a test compound;
- and,

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- (d) monitoring fluorescence of the cell cytosol and/or plasma membrane during incubation in (b) or (c),

wherein a difference in fluorescence in the cytosol and/or at the plasma membrane in the presence of the test compound compared with that in the absence of the test compound is indicative that the test compound modulates calcium ion influx.

22. A method for identifying a compound that agonises calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing FP-CAPPI or a FP-CAPPI derivative, which is capable of translocation to and association with the plasma membrane,

- (b) incubating the eukaryotic cell in the presence of a test compound and

(c) monitoring fluorescence of the cell cytosol and/or plasma membrane wherein a decrease in cytosolic fluorescence, and/or increase in plasma membrane fluorescence following addition of the test compound is indicative that the test compound agonises calcium ion influx.

23. A method for identifying a compound that antagonises calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing FP-CAPPI or a FP-CAPPI derivative which is capable of translocation to and association with the plasma membrane,

- (b) incubating the eukaryotic cell and providing a stimulus for calcium ion influx,

- (c) monitoring fluorescence of the cell cytosol and/or plasma membrane,

- (d) introducing a test compound to the incubation mixture,

(e) monitoring fluorescence of the cell cytosol and/or plasma membrane, wherein an increase in cytosolic fluorescence and/or a decrease in plasma membrane fluorescence following addition of the test compound is indicative that the test compound antagonises calcium ion influx.

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24. A method for identifying a compound that agonises calcium ion influx comprising:

- (a) providing a eukaryotic cell expressing FP-CAPRI or a FP-CAPRI derivative which is capable of translocation to and association with the plasma membrane,

(b) incubating the cell with the test compound for a predetermined time,

(c) monitoring fluorescence of the cell cytosol and/or plasma membrane, wherein a decrease in cytosolic fluorescence and/or an increase in plasma membrane fluorescence following addition of the test compound is indicative that the test compound agonises calcium ion influx.

25. A method according to any one of claims 20 to 24 wherein the fluorescent marker is a quantum dot.

26. A method according to any one of claims 20 to 24 wherein the fluorescent marker is a fluorescent protein.

27. A method according to claim 26 wherein the fluorescent protein is a red, orange, yellow, yellow-green, green, yellow-green, green, yellow-green, or blue fluorescent protein.

28. A method according to claim 26 wherein the fluorescent protein is a green fluorescent protein (GFP).

29. A method according to claim 27 or 28 wherein the fluorescent protein is a wild type, enhanced, destabilised enhanced, or red-shift fluorescent protein.

30. A method according to any one of claims 20 to 29, wherein monitoring is performed by fluorescence microscopy.

31. A method according to claim 30, wherein fluorescence microscopy is performed by wide-field or total internal reflection fluorescence microscopy or fluorescence lifetime imaging or confocal imaging.

32. A method according to any one of claims 20 to 31, wherein monitoring is performed by measuring fluorescence at the region(s) of interest within the cell over time

33. A method according to claim 32 wherein the translocation of the reporter with the plasma membrane and/or distribution of reporter in the cytosol is assessed by calculating the relative translocation parameter $(1 - F_{\text{cyt}}/F_0)$ at one or more time points, wherein F_0 is the fluorescence in a region(s) of interest (e.g. cytosol and/or plasma membrane) at the start of monitoring and F_t is fluorescence in a region(s) of interest at a particular time point.

34. A method according to claim 33 wherein monitoring is performed by measuring cytosolic fluorescence over time and calculating the relative translocation parameter at one or more time points, $1 - F_{\text{cyt}}/F_{0\text{cyt}}$, wherein $F_{0\text{cyt}}$ is the cytosolic fluorescence in the region of interest at the start of monitoring and $F_{t\text{cyt}}$ is the cytosolic fluorescence in the region of interest at a particular time point.

35. A method according to any preceding claim, wherein the data is in a digital format.

ABSTRACT:

A method for detecting influx of calcium ions into a eukaryotic cell comprising providing a eukaryotic cell having a detectable reporter capable of translocation from the cytosol to associate with the plasma membrane in response to an increase in intracellular calcium ion concentration. The detectable reporter is in the cytosol and, in response to an increase in intracellular calcium ion concentration, translocates with the plasma membrane and/or associates in the cytosol. The reporter in the cytosol. The detectable reporter is preferably C/EBP or a derivative thereof and is preferably labelled with a fluorescent marker.



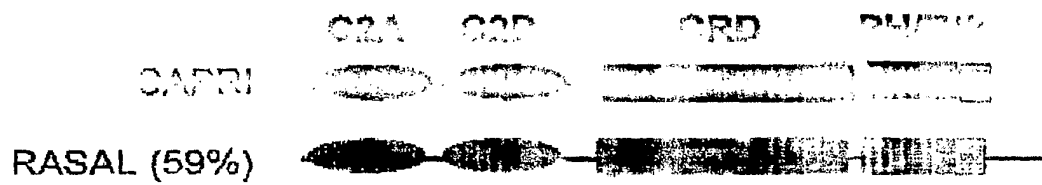
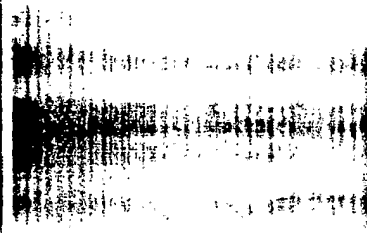
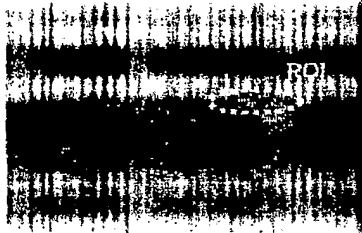
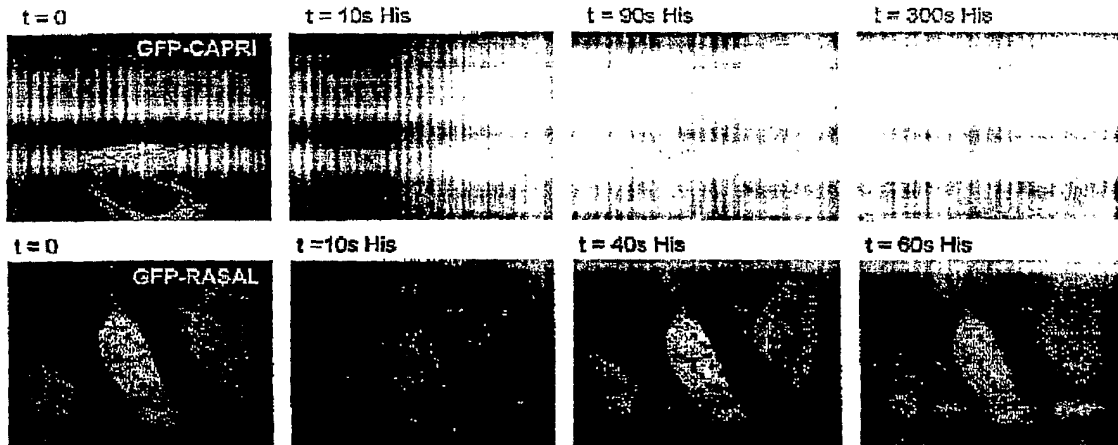


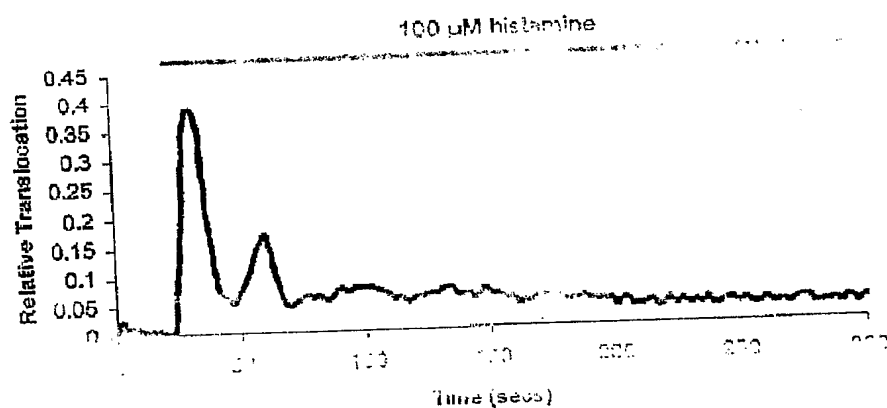
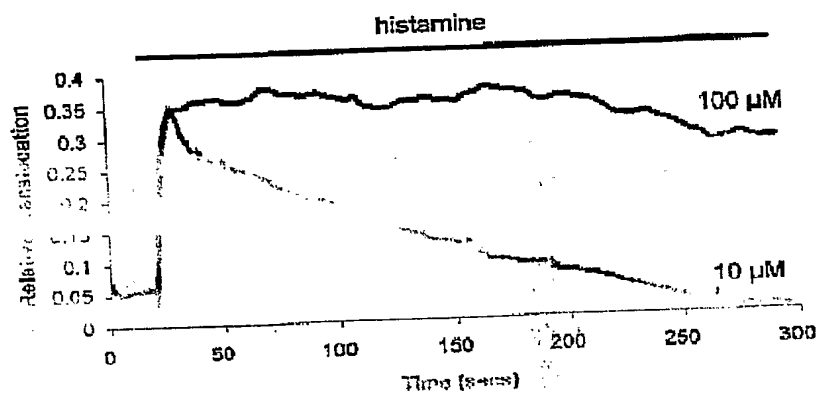
Figure 1



A F_0 = maximum cytosolic fluorescence F_t = cytosolic fluorescence at given time pointRelative Translocation
 $= 1 - F_t/F_0$ **B****Figure 2**



C



D

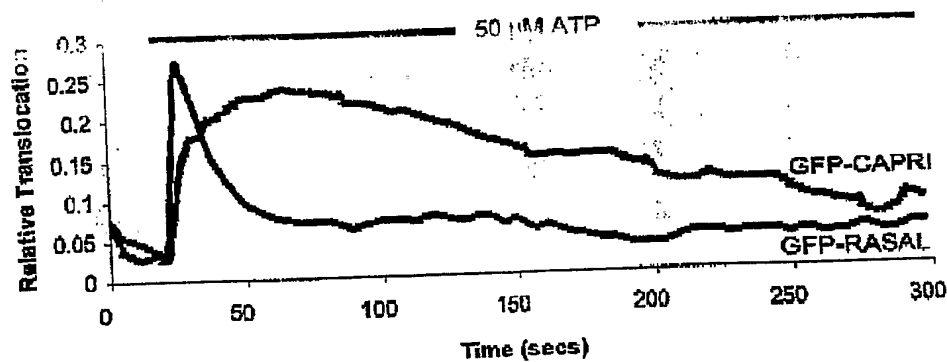


Figure 2



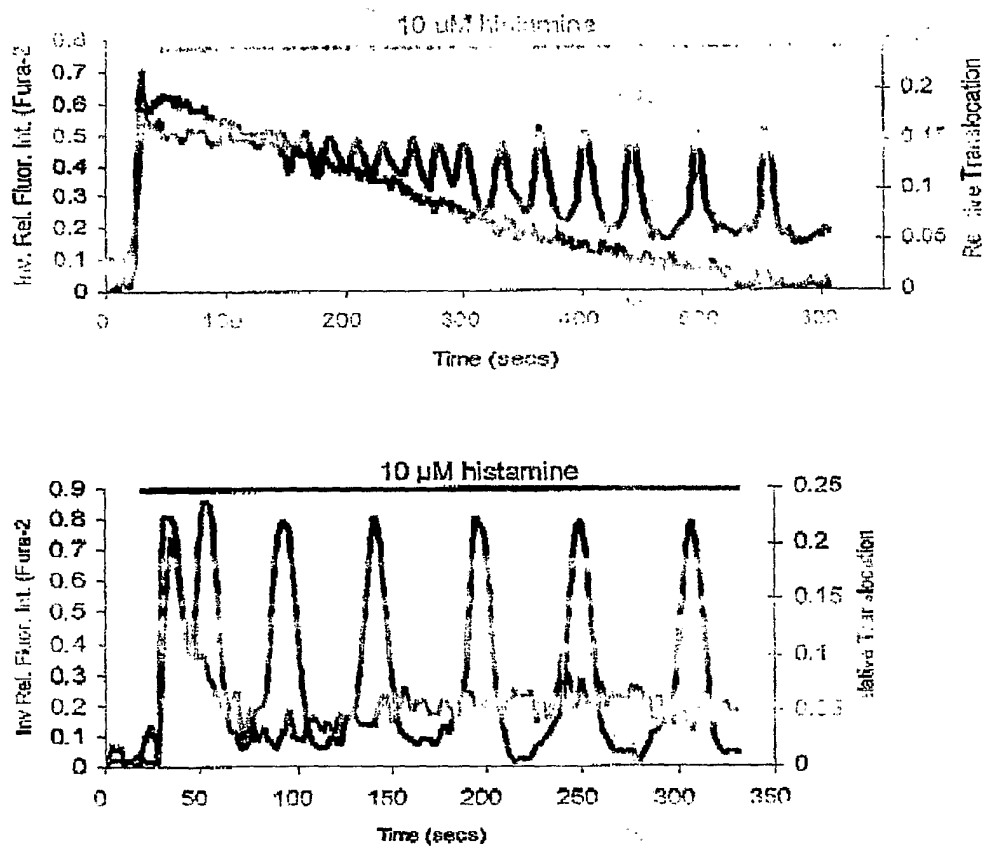


Figure 3



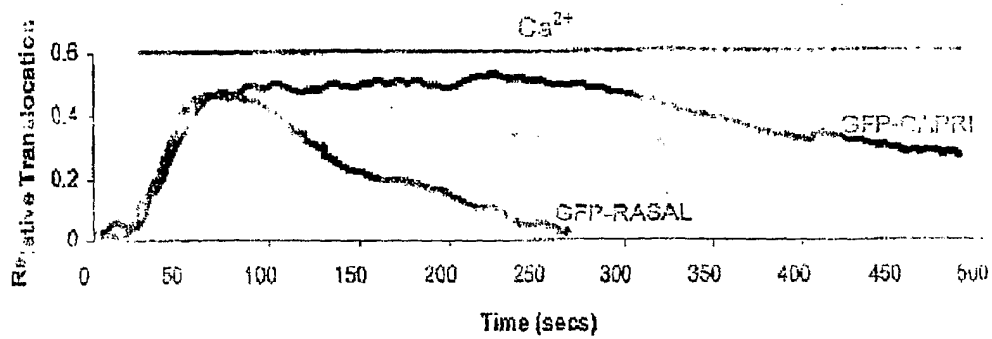


Figure 4

